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YALI0E32769g (*DGA1*) and YALI0E16797g (*LRO1*) encode major triacylglycerol synthases of the oleaginous yeast *Yarrowia lipolytica*

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ABSTRACT

The oleaginous yeast *Yarrowia lipolytica* has an outstanding capacity to produce and store triacylglycerols resembling adipocytes of higher eukaryotes. Here, the identification of two genes YALI0E32769g (*DGA1*) and YALI0E16797g (*LRO1*) encoding major triacylglycerol synthases of *Yarrowia lipolytica* is reported. Heterologous expression of either *DGA1* or *LRO1* in a mutant of the budding yeast *Saccharomyces cerevisiae* defective in triacylglycerol synthesis restores the formation of this neutral lipid. Whereas Dga1p requires acyl-CoA as a substrate for acylation of diacylglycerol, Lro1p is an acyl-CoA independent triacylglycerol synthase using phospholipids as acyl-donor. Growth of *Yarrowia lipolytica* strains deleted of *DGA1* and/or *LRO1* on glucose containing medium significantly decreases triacylglycerol accumulation. Most interestingly, when oleic acid serves as the carbon source the ratio of triacylglycerol accumulation in mutants to wild-type is significantly increased in strains defective in *DGA1* but not in *lro1Δ*. In vitro experiments revealed that under these conditions an additional acyl-CoA dependent triacylglycerol synthase contributes to triacylglycerol synthesis in the respective mutants. Taken together, evidence is provided that *Yarrowia lipolytica* contains at least four triacylglycerol synthases, namely Lro1p, Dga1p and two additional triacylglycerol synthases whereof one is acyl-CoA dependent and specifically induced upon growth on oleic acid.

1. Introduction

Major neutral lipids occurring in most eukaryotic cells are triacylglycerols (TAG) and steryl esters. These lipids accumulate when cells are provided with an excess of nutrients. During starvation periods these storage lipids are mobilized serving as a source of energy and/or building blocks for the formation of membrane lipids. Due to the importance of TAG as an inert storage molecule, its metabolism appears to be conserved from bacteria to men. Synthesis of TAG occurs by acylation of the free hydroxyl-group of its direct precursor diacylglycerol. This final step of TAG formation is either catalyzed by an acyl-CoA dependent or acyl-CoA independent enzyme (Fig. 1, for reviews see [1,2]).

In the yeast *Saccharomyces cerevisiae* Lro1p has been identified as an acyl-CoA independent acyltransferase [3,4]. This enzyme is a homolog of the human lecithin:cholesterol acyltransferase (LCAT). Similar to its human counterpart, Lro1p cleaves off an acyl group from the *sn*-2 position of phosphatidylcholine or phosphatidylethanolamine, but uses diacylglycerol as the acceptor molecule instead. Lro1p is the only acyl-CoA independent acyltransferase of *S. cerevisiae*, since an acyl-CoA independent activity has not been detected in the respective deletion mutant. In contrast, three enzymes are known to catalyze acylation of DAG yielding TAG via the acyl-CoA dependent pathway. The acyl-CoA: diacylglycerol acyltransferase Dga1p is the major acyl-CoA dependent TAG synthase in *S. cerevisiae* [5–7]. Dga1p is a member of the DGAT2 family. Are1p and Are2p, two ACAT (acyl-CoA:cholesterol acyltransferase) related enzymes originally considered to acylate specifically sterols, have been demonstrated to be responsible for the residual acyl-CoA dependent TAG synthase activity in an *lro1Δdga1Δ* double mutant [6,8]. This is not surprising insofar, since Are1p and Are2p are members of the DGAT1 family, containing the mammalian DGAT1 and acyl-CoA: cholesterol acyltransferases of plants and mammals. Deletion of *ARE1* and *ARE2* in an *lro1Δdga1Δ* mutant background results in a quadruple mutant, i.e., *lro1Δdga1Δare1Δare2Δ*, lacking TAG. This fact indicates that in contrast to plants and mammalian cells, in *S. cerevisiae* no enzyme exists which catalyzes the transacylase reaction converting two diacylglycerol molecules to TAG and monoacylglycerol [9,10].

In the fission yeast *Schizosaccharomyces pombe* Plh1p, an acyl-CoA independent TAG synthase, and Dga1p, catalyzing the acyl-CoA dependent acylation of diacylglycerol, have been identified by gene and function [11]. Plh1p and Dga1p of the fission yeast are the respective counterparts of Lro1p and Dga1p of *S. cerevisiae*. Whereas a defect in either *LRO1* or *DGA1* of the budding yeast is reflected by a decreased amount of TAG [7], no difference in the accumulation of TAG compared to control has been detected in the single deletion mutants *plh1Δ* and *dga1Δ* of *S. pombe* [11]. This finding reveals that in the latter yeast one TAG synthase compensates the defect/lack of the

Abbreviation: TAG, triacylglycerols

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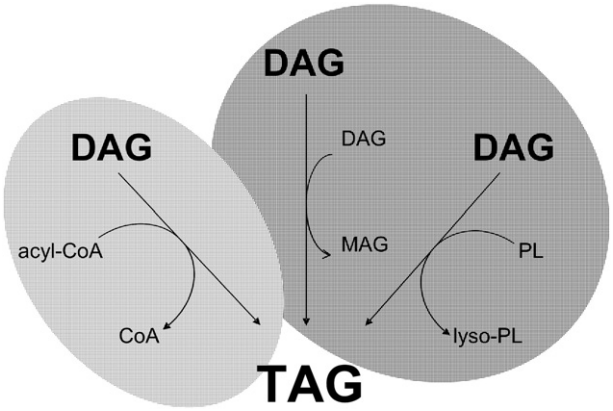

Fig. 1. Mechanisms of triacylglycerol synthesis. Triacylglycerols (TAG) are either formed via an acyl-CoA dependent (light gray) or acyl-CoA independent (dark gray) mechanism. Abbreviations: DAG: diacylglycerol; MAG: monoacylglycerol; PL: phospholipid.

other enzyme. Plh1p and Dga1p are the only TAG synthases of *S. pombe,* since the *plh1Δdga1Δ* double mutant lacks TAG.

In the oleaginous yeast *Yarrowia lipolytica* mainly TAG and only minor quantities of steryl esters accumulate as storage lipids [12]. Like in all eukaryotic cells these neutral lipids are sequestered from the cytosolic environment in so-called lipid particles. The structure of these storage compartments of neutral lipids is rather simple. TAG and steryl esters are forming a hydrophobic core which is surrounded by a phospholipid monolayer with few proteins embedded (for a review see [13]). Under specific growth conditions, e.g., when industrial fats or glycerol are used as a carbon source, the amount of TAG can be increased up to 40% of cell dry weight [14]. Microscopic inspection of cells grown under such conditions reveals that nearly the entire cell is filled with lipid particles [15]. This outstanding capacity of *Y. lipolytica* to accumulate neutral lipids (mainly TAG) leads to its application in biotechnological processes such as single cell oil production or production of nutrients enriched in essential fatty acids which can serve as nutritional complements. However, *Y. lipolytica* may also serve as a model organism for studying lipid turnover in adipocytes, since not only the ability to store excessive amounts of TAG in lipid particles but also the composition of this cell compartment resembles adipocytes of higher eukaryotes. Despite these potentials of *Y. lipolytica* current information about TAG (lipid) metabolism in this yeast is rather limited.

Here, evidence is provided that the gene products of YALI0E32769g (*DGA1*) and YALI0E16797g (*LRO1*) are major TAG synthases of the oleaginous yeast *Y. lipolytica*. This conclusion is based on results from analyses of lipid profiles and measurements of enzymatic activities of wild-type, the single deletion strains *lro1Δ* and *dga1Δ*, and the double deletion mutant *lro1Δdga1Δ*. Most importantly, heterologous expression of the respective genes in cells of a *S. cerevisiae* mutant defective in TAG synthesis complements the defect of the host cells. Furthermore, the results reveal that growth on oleic acid containing medium but not on glucose containing medium induces acyl-CoA dependent TAG synthase activity in a *Y. lipolytica* mutant lacking *DGA1* and *LRO1*.

2. Materials and methods

2.1. Strains and culture conditions

Yeast strains used throughout this study are listed in Table 1. *Y. lipolytica* cells were grown aerobically in Erlenmeyer flasks at 30 °C in medium containing 0.67% yeast nitrogen base (US Biological), 0.1% yeast extract (Oxoid), 0.5% NH_4Cl (Merck), 50 mM potassium phosphate buffer (pH 6.8), 0.01% leucine (Roth) and either 2% glucose (Merck) or 1% oleic acid (Merck) dissolved with 1% Brij58 as a carbon source. Fifty milliliters of culture medium were inoculated to an optical density (OD_{600}) of 0.01 from an over night culture grown aerobically. Growth was monitored by measuring OD_{600}.

For heterologous expression studies *S. cerevisiae* cells transformed with plasmids containing either *LRO1* or *DGA1* of *Y. lipolytica* were grown aerobically in Erlenmeyer flasks at 30 °C in synthetic minimal (SM) media containing 2% glucose or 2% galactose (Merck), respectively, and 0.67% yeast nitrogen base (US Biological) supplemented with the appropriate amino acids.

Table 1
Strains and plasmids used in this study.

Strain	Genotype	Reference
Yarrowia lipolytica		
JMY330 (wild-type)	*MATA leu2-270 xpr2-322*	Kindly provided by J.-M. Nicaud
JMY1201 (*lro1Δ*)	*JMY330; lro1Δ::URA3*	Kindly provided by J.-M. Nicaud
JMY1204 (*dga1Δ*)	*JMY330; dga1Δ::URA3*	Kindly provided by J.-M. Nicaud
JMY1281 (*lro1Δdga1Δ*)	*JMY330; lro1Δ::URA3; dga1Δ*	Kindly provided by J.-M. Nicaud
Saccharomyces cerevisiae		
BY4741 + pYES2 (wild-type control)	*Mat* **a**; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0;* + pYES2	This study
lro1Δdga1Δare1Δare2Δ + pYES2	BY4741; *Mat* **a**; *lro1Δ::KanMX4; dga1Δ::KanMX4; are1Δ::KanMX4; are2Δ::KanMX4* + pYES2	This study
lro1Δdga1Δare1Δare2Δ + pYES2-*LRO1*^{YI}	BY4741; *Mat* **a**; *lro1Δ::KanMX4; dga1Δ::KanMX4; are1Δ::KanMX4; are2Δ::KanMX4* + pYES2-*LRO1*^{YI}	This study
lro1Δdga1Δare1Δare2Δ + pYES2-*DGA1*^{YI}	BY4741; *Mat* **a**; *lro1Δ::KanMX4; dga1Δ::KanMX4; are1Δ::KanMX4; are2Δ::KanMX4* + pYES2-*DGA1*^{YI}	This study
Plasmids	**Description**	
pYES2		Invitrogen
pYES2-*LRO1*^{YI}	pYES2 containing *LRO1* of *Yarrowia lipolytica*	This study
pYES2-*DGA1*^{YI}	pYES2 containing *DGA1* of *Yarrowia lipolytica*	This study

2.2. Genetic techniques

For heterologous expression of either Dga1p or Lro1p of *Y. lipolytica* in the quadruple deletion mutant *lro1Δdga1Δare1Δare2Δ* of the budding yeast *S. cerevisiae* the respective open reading frames YALI0E32769g (*DGA1*) and YALI0E16797g (*LRO1*) were inserted into the pYES2 vector (Invitrogen). Insertion cassettes were obtained by PCR using genomic DNA derived from a *Y. lipolytica* wild-type strain as template. Primers used for amplification are shown in Table 2. PCR products were purified with NucleoSpin purification kit (Macherey-Nagel). The plasmid was cleaved with *Bam*HI and *Eco*RI. PCR products and the cleaved plasmid were purified by agarose gel electrophoresis and fragments recovered from the gel with NucleoSpin gel extraction kit (Macherey-Nagel). The cleaved plasmid and the purified PCR fragment of either *DGA1* or *LRO1* were used for transformation of the quadruple deletion mutant *lro1Δdga1Δare1Δare2Δ* of *S. cerevisiae*. The respective plasmids were formed upon homologous recombination. The correct insertion of the PCR fragments into the plasmid was verified by colony PCR with control primers listed in Table 2.

Table 2
Primers used for plasmid construction. Underlined sequences are homologous to the respective ORF or the downstream uncoding region.

Primer	Sequence 5′→ 3′
LRO1^{YI} (fw)	CTAGCAGCTGTAATACGACTCACTATAGGGAATATTAAGCTTGGTACCGAGCTCGGATCCACTATGGCACAACCTGTGAATCGGA
LRO1^{YI} (rev)	ATGCGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCCCCTTCTCCATCAGTGTG
DGA1^{YI} (fw)	CTAGCAGCTGTAATACGACTCACTATAGGGAATATTAAGCTTGGTACCGAGCTCGGATCCACTATGGCTATCGACTCACAATACT
DGA1^{YI} (rev)	ATGCGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCATACTCATTAGCTATTTGC
GAL1 (fw; control)	TGCATAACCACTTTAACT
LRO1^{YI} (rev; control)	CCTTGCCCACAGCAAAAG
DGA1^{YI} (rev; control)	CGTTGATAGAAAGAGACCG

The quadruple mutant *lro1Δdga1Δare1Δare2Δ* of the budding yeast *S. cerevisiae* was obtained by crossings and tetrad dissection of the respective single deletion strains (EUROSCARF strain collection). The disruption of all four genes in the respective mutant was verified by control PCR and confirmed by the absence of TAG and steryl esters resulting in a lack of lipid particles (see below).

2.3. Preparation of enzyme sources for activity measurements

Homogenates and microsomes of the strains listed in Table 1 were isolated as described previously [16]. Strains of *Y. lipolytica* grown on oleic acid containing media were washed three times with 0.5% BSA solution prior to application of the protocol for spheroplast and organelle preparation. Spheroplasts were prepared by treatment with zymolyase as described by Daum et al. [17]. Microsomal fractions were obtained by following the protocol of Zinser et al. [16].

For protein quantification polypeptides were precipitated from the aqueous phase with trichloroacetic acid at a final concentration of 10%. The protein pellet was solubilized in 0.1% SDS – 0.1 M NaOH at 37 °C. Protein quantification was performed by the method of Lowry et al. [18] using bovine serum albumin as a standard.

2.4. Lipid analysis

Total lipids of cells grown to the stationary phase (24 h) were extracted by the procedure of Folch et al. [19]. Strains of *Y. lipolytica* grown in the presence of oleic acid were washed three times with 0.5% BSA solution prior to lipid extraction. Analysis of neutral lipids was performed as described in [20]. In brief, lipids were separated by thin-layer chromatography (TLC) using the solvent systems petroleum ether-diethyl ether-acetic acid (25:25:1; per vol.; developed to 1/3 of the total distance) and petroleum ether-diethyl ether (49:1; vol./vol.; total distance). Neutral lipids were visualized on TLC plates by post-chromatographic staining. Plates were dipped for 6 s into a developing reagent consisting of 0.63 g of $MnCl_2.4H_2O$, 60 ml of water, 60 ml of methanol, and 4 ml of concentrated sulphuric acid, briefly dried, and heated to 105 °C for 30 min. Subsequently, TAG were quantified by densitometric scanning at 400 nm with triolein (NuCheck, Inc., Elysian, MN) as a standard.

2.5. Fluorescence microscopy

Y. lipolytica cells were grown either on glucose or on oleic acid containing medium for 24 h prior to microscopic inspection. To analyze the functionality of *DGA1* and *LRO1*, respectively, upon heterologous expression in the *S. cerevisiae* quadruple mutant *lro1Δdga1Δare1Δare2Δ* transformants were grown to the stationary phase in Ura$^-$ minimal medium containing either glucose or galactose as the carbon source. Cells were harvested by centrifugation washed twice and put onto microscope slides. By mixing the cell suspension with a solution of 1 mg/ml Nile Red® (Sigma) in ethanol, cells were stained directly on the slide. Microscopic analysis was performed on a Zeiss Axiovert 35 microscope using a 100-fold oil immersion objective, a UV lamp, and a detection range between 450 and 490 nm. Images were taken with a CCD camera.

2.6. Enzyme assays

The diacylglycerol acyltransferase assay was performed as described previously [7]. In brief, enzymatic activity was measured in a final volume of 400 µl containing 10 nmol dipalmitoylglycerol, 100 nmol unlabeled (Sigma) and radioactively labeled [1-^{14}C]oleoyl-CoA or [1-^{14}C]palmitoyl-CoA (0.04 µCi; Perkin Elmer), 400 µg BSA, 30 µg dithiothreitol, 0.25% Triton X-100, 150 mM Tris/Cl$^-$ (pH 7.0), 15 mM KCl, 15 mM $MgCl_2$ and either homogenate (0.7 to 0.9 mg) or microsomes (0.1 to 0.3 mg) as the respective enzyme source. For measuring the phospholipid:diacylglycerol acyltransferase activity the assay mixture was essentially the same as for the diacylglycerol acyltransferase assay but the amount of dipalmitoylglycerol was increased to 20 nmol, [^{14}C]phospholipids (75,000 dpm; approx. 25 µg) were used as the labeled substrate and acyl-CoA was omitted. Both assays were carried out at 30 °C and the reaction was terminated after 30 min by the addition of 3 ml chloroform-methanol (2:1; vol./vol.). Lipids were extracted [19], separated by thin-layer chromatography with the solvent system petroleum ether-diethyl ether-acetic acid (70:30:2; per vol.), scraped off the plate and radioactivity was measured by liquid scintillation counting in 8 ml liquid-scintillation cocktail containing 5% water.

Radioactively labeled phospholipids were synthesized by incubation of a *S. cerevisiae* strain with 0.1 mCi of [1-^{14}C]palmitic acid for 24 h at 30 °C in 100 ml medium containing 2% glucose (Merck), 1% yeast extract (Oxoid) and 2% peptone (Oxoid). Lipids were extracted by using the method of Folch et al. [19] and separated by thin-layer chromatography. The band containing phospholipids was scraped off the plate and phospholipids extracted from the silica gel with chloroform-methanol (1:4; vol./vol.). Label-distribution between major glycerophospholipids: phosphatidylcholine 45%, phosphatidylethanolamine 30%, phosphatidylinositol 14%, phosphatidylserine 11%.

3. Results

3.1. Two open reading frames (ORFs) of Yarrowia lipolytica homologous to genes encoding TAG synthases

Analysis of the lipid particle proteome of *Y. lipolytica* [12] revealed the presence of a protein encoded by the open reading frame YALI0E32769g sharing 50% identity and 71% homology to the acyl-CoA:diacylglycerol acyltransferase Dga1p of the budding yeast *S. cerevisiae* and 51% identity and 72% homology to the respective enzyme in the fission yeast *S. pombe* (blast search: http://www.ch.embnet.org). In addition, the sequence of the potential TAG synthase of *Y. lipolytica* reveals homology to acyl-CoA:diacylglycerol acyltransferases of mammals, e.g., mouse, rat and human (56% homology). The predicted molecular mass of the Dga1p homolog of *Y. lipolytica* is 57.8 kDa (514 amino acids) with a pI of 8.7 (http://www.ch.embnet.org/software/

TMPRED_form.html). Noteworthy, this protein contains after the first ~120 amino acids an insertion of 113 and 155 amino acids, respectively, compared to Dga1p of *S. cerevisiae* and *S. pombe* (Fig. 2A).

The second major TAG-synthase of the budding yeast *S. cerevisiae* is Lro1p (lecithin:cholesterol acyltransferase (LCAT) related ORF). Homology searches in the genome of *Y. lipolytica* with *LRO1* of the budding yeast as a query highlighted the ORF YALI0E16797g as a sequence encoding a potential acyl-CoA independent TAG synthase. The respective protein encoded by YALI0E16797g shares 51% identity and 68% homology to Lro1p of *S. cerevisiae*, and 46% identity and 64% homology to Plh1p, the Lro1p counterpart of *S. pombe* (Fig. 2B). Furthermore, the potential TAG synthase of the oleaginous yeast is related to lecithin:cholesterol acyltransferases (LCAT) of mammals, e.g., rabbit, mouse, human. The Lro1p homolog of *Y. lipolytica* is formed of 648 amino acids with an estimated molecular mass of 72.6 kDa and a pI of 5.5 (http://www.ch.embnet.org/software/TMPRED_form.html).

For both potential TAG synthases of the oleaginous yeast a transmembrane domain is predicted at the N-terminus (Fig. 2C). Similarly, Dga1p and the phospholipid:diacylglycerol acyltransferase of the budding and fission yeast, respectively, contain an N-terminal transmembrane domain (Kyte Doolittle blot: http://fasta.bioch.virginia.edu/).

Due to the homologies of the respective gene products to already identified TAG synthases of other eukaryotes these unassigned genes of *Y. lipolytica* are named hereafter *DGA1* (YALI0E32769g) and *LRO1* (YALI0E16797g).

3.2. The number and size of lipid particles is changed in lro1Δdga1Δ cells

Since TAG and steryl esters lack charged groups they do not fit into the phospholipid bilayer of membranes and are sequestered from the cytosolic environment in the hydrophobic core of so-called lipid particles. In the oleaginous yeast *Y. lipolytica* the hydrophobic core of lipid particles consists mainly of TAG [12]. To analyze whether deletion of *DGA1* and/or *LRO1* affects the size and/or number of lipid particles, cells grown to the stationary phase were stained with the fluorescent dye NileRed® specific for these compartments (see Materials and methods). Whereas the number and size of lipid particles in cells of the single deletion mutants *lro1Δ* and *dga1Δ* grown on glucose containing medium are similar to control, a higher number of small lipid particles is formed in the double deletion mutant *lro1Δdga1Δ* (Fig. 3). In contrast, no significant differences in the formation of lipid particles are observed between the single deletion mutants *lro1Δ* and *dga1Δ*, the double

A

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Dga1 (Sc)       MSGTFNDIRRRKKEEGSPTAGITERHENKSLSSIDKREQTLKPQLESCCPLATPFERRLQ
Dga1 (Sp)       -------------------------MSEETSIPGIIASTPPIS----KDSRRNVSHWLQ
Dga1 (Yl)       ----------------------MTIDSQYYKSRDKNDTAPKIAGIRYAPLSTPLLNRCE
                                      .:  .  .    :              .  .  :

seqA            TLAVAWHTSSFVLFSIFTLFAISTPALWVLAIPYMIYFFFDRSPATGEVVNRYSLRFRSL
seqB            ALAVFLHSVSLTLTASWYTVLWAFLPFWPFLIVYLIWLIYDDGFVTG--KDRQKRWLRNA
seqC            TFSLVWHIFSIPTFLTIFMLCCAIPLLWPFVIAYVVYAVKDDSPSNGGVVKRYSPISRNF
                ::::  *  *:        .  :   :* : * *::: . * .  .*   .* .   *.

seqA            PIWKWYCDYFPISLIKTVNLKPTFTLSKNKRVNEKNYKIRLWPTKYSIN-----------
seqB            PPYRWFCHYFPIRLHKTTELD--------------------------------------
seqC            FIWKLFGRYFPITLHKTVDLEPTHTYYPLDVQEYHLIAERYWPQNKYLRAIISTIEYFLP
                  :: :  **** * **.:*.

seqA            ------------------------------------------------------------
seqB            ------------------------------------------------------------
seqC            AFMKRSLSINEQEQPAERDPLLSPVSPSSPGSQPDKWINHDSRYSRGESSGSNGHASGSE


seqA            -----------------------------------------LKSNSTIDYRNQECTGPT
seqB            ------------------------------------------------------SEKN
seqC            LNGNGNNGTTNRRPLSSASAGSTASDSTLLNGSLNSYANQIIGENDPQLSPTKLKPTGRK
                                                                      :  .

seqA            YLFGYHPHGIGALGAFGAFATEGCNYSKIFPGIPISLMTLVTQFHIPLYRDYLLALGISS
seqB            YIFGYHPHGIISLGAFGGFASEGADFSKLFPGINVSVLTLNSNFYVPVYRDYLMALNINS
seqC            YIFGYHPHGIIGMGAFGGIATEGAGWSKLFPGIPVSLMTLTNNFRVPLYREYLMSLGVAS
                *:******** .:****.:*:**..:**:**** :*::** .:* :*:**:**::*.: *

seqA            VSRKNALRTLSKN--QSICIVVGGARESLLSSTNGTQLILNKRKGFIKLAIQTGNINLVP
seqB            VSKKSCVSILSRKPGDSVLIVIGGAQESLLSRPGQNNLVLKKRFGFVKLAFLTG-SSLVP
seqC            VSKKSCKALLKRN--QSICIVVGGAQESLLARPGVMDLVLLKRKGFVRLGMEVGNVALVP
                **:*..   *.::  :*: **:***:****: ..  :*:* ** **::*.: .*   ***

seqA            VFAFGEVDCYNVLSTKKDSVLGKMQLWFKENFGFTIPIFYARGLFNYDFGLLPFRAPINV
seqB            CFAFGESDIFEQVDNNPRTRIYKFQEIVKKIAGFTVPFFYGRGLLNKTFGLMPWRKPINI
seqC            IMAFGENDLYDQVSNDKSSKLYRFQQFVKNFLGFTLPLMHARGVFNYDVGLVPYRRPVNI
                 :**** * :: :...  : : ::*  .*:  ***:*:::.**::*  .**:*:* *:*:

seqA            VVGRPIYVEKKITNPPDDVVNHFHDLYIAELKRLYYENREKYGVPDAELKIVG-------
seqB            VVGEPIDVPKKS-HPTNQEIYEVHEEYIRRLEGLWNKYKDVFLPNRISELKLSA------
seqC            VVGSPIDLPYLP-HPTDEEVSEYHDRYIAELQRIYNEHKDEYFIDWTEEGKGAPEFRMIE
                *** ** :     :*.:: : . *: ** .*: :: : :: :     .    .
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Fig. 2. Sequence alignments and hydropathy plots of triacylglycerol synthases. Multiple alignments of acyl-CoA:diacylglycerol acyltransferases (A) and the lecithin:cholesterol acyltransferase (LCAT) related proteins (B) of the budding yeast *Saccharomyces cerevisiae* (SeqA), the fission yeast *Schizosaccharomyces pombe* (SeqB) and the oleaginous yeast *Yarrowia lipolytica* (SeqC). "*": identical amino acid in all proteins; ":" identical amino acid in two polypeptides; "." homologous amino acid. Program: http://www.ch.embnet.org/. Hydropathy plots of Dga1p and Lro1p of *Yarrowia lipolytica* are shown in panel C. Dga1p contains an insert of 113 (155) amino acids (marked by the black line) after the N-terminal transmembrane domain which is not present in the respective counterparts of the budding yeast and the fission yeast. Program: http://fasta.bioch.virginia.edu/.

B

```
Lro1p (Sc)      -MGTLFRRNVQNQKSDSDENNKGGSVHNKRESRNHIHHQQGLGHKRRRGISGSAKRNERG
Plh1p (Sp)      --MASSKKSKTHKKKKEVKSPIDLPNSKKPTRALSEQPSASETQSVSNKSRKSKFGKRLN
Lro1p (Yl)      MTQPVNRKATVERVEPAVEVADSESEAKTDVHVHHHHHHHKRKSVKGKILNFFTRSRRIT
                   .  ::   .: .   :   . .  :.       :          .        ..

seqA            KDFDRKRDGNGRKRWRDSRRLIFILGAFLGVLLPFSFGAYHVHNSDSDLFDNFVNFDSLK
seqB            FILGAILGICGAFFFAVGDDNAVFDPATLDKFG--------------------NMLGSS
seqC            FVLGAVVGVIAAGYYAAPPELSIDIDALLGDLPSFDFDALSLDN---------LSMDSVS
                  :.   .  .   :       .   * *. :                     .: . .

seqA            VYLDDWKDVLPQGISSFIDDIQAGNYSTSSLDDLSENFAVGKQLLRDYNIEAKHPVVMVP
seqB            DLFDDIKGYLSYNVFKDAPFTTDKPSQSP----SGNEVQVGLDMYNEG-YRSDHPVIMVP
seqC            DFVQDMKSRFPTKILQEAAKIEKHQKSEQ----KAAPFAVGKAMKSEG-LNAKYPVVLVP
                  .:* *. :.  : .          .       .  . **  :  :   .:.:**::**

seqA            GVISTGIESWGVIGDDECDSSAHFRKRLWGSFYMLRTMVMDKVCWLKHVMLDPETGLDPP
seqB            GVISSGLESWSFNN----CSIPYFRKRLWGSWSMLKAMFLDKQCWLEHLMLDKKTGLDPK
seqC            GVISTGLESWSLEGTEECPTESHFRKRMWGSWYMIRVMLLDKYCWLQNLMLDTETGLDPP
                ****:*:***.. .     : .:****:***: *::.*.:** ***:::*** :*****

seqA            NFTLRAAQGFESTDYFIAGYWIWNKVFQNLGVIGYEPNKMTSAAYDWRLAYLDLERRDRY
seqB            GIKLRAAQGFEAADFFITGYWIWSKVIENLAAIGYEPNNMLSASYDWRLSYANLEERDKY
seqC            HFKLRAAQGFASADFFMAGYWLWNKLLENLAVIGYDTDTMSAAAYDWRLSYPDLEHRDGY
                 :.******* ::*:*::***:*.*:::**..***:.:.* :*:*****:* :**.** *

seqA            FTKLKEQIELFHQLSGEKVCLIGHSMGSQIIFYFMKWVEAEGPLYGNGGRGWVNEHIDSF
seqB            FSKLKMFIEYSNIVHKKKVVLISHSMGSQVTYYFFKWVEAEG--YGNGGPTWVNDHIEAF
seqC            FSKLKASIEETKRMTGEKTVLTGHSMGSQVIFYFMKWAEAEG--YGGGGPNWVNDHIESF
                *:***  **  : :  :*. * .******: :**:**.****  **.**  ***:**::*

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seqB            INISGSLIGAPKTVAALLSGEMKDTGIVITLNIL--EKFFSRSERAMMVRTMGGVSSMLP
seqC            VDISGSMLGTPKTLVALLSGEMKDTVQLNAMAVYGLEQFFSRRERADLLRTWGGIASMIP
                :: :*:::*:**:: **:*******  : :: :   *:**** **. :::* **:.**:*

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seqC            KGGKAIWGDHSGAPDDEPGQ-NVTFGNFIKFKESLTEYSAKNLTMDETVDFLYSQSPEWF
                ** ..  .* . :            .. *   ..  :   .:: :.:::::  . : : :

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seqB            KVMLAKNYSHGLAWTEKEVLKNNEMPSKWINPLETSLPYAPDMKIYCVHGVGKPTERGYY
seqC            VNRTEGAYSFGIAKTRKQVEQNEKRPSTWSNPLEAALPNAPDLKIYCFYGVGKDTERAYY
                       **.* : ..::: :*:   . * **:*..** **.:****.:**.: ***.*

seqA            YKEEDDSS-ALNLTIDYESKQP--VFLTEGDGTVPLVAHSMCHKWAQGASPYNPAGINVT
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seqC            YQDEPNPE-QTNLNVSIAGNDPDGVLMGQGDGTVSLVTHTMCHRWKDENSKFNPGNAQVK
                * :: : .   : .:.   :    :.: :****:.:::  :  .     . :**.. .:.

seqA            IVEMKHQPDRFDIRGGAKSAEHVDILGSAELNDYILKIASGNGDLVEPRQLSNLSQWVSQ
seqB            NYEIKHEPAAFDLRGGPRSAEHVDILGHSELNEIILKVSSGHGDSVPNRYISDIQEIINE
seqC            VVEMLHQPDRLDIRGGAQTAEHVDILGRSELNEMVLKVASGKGNEIEERVISNIDEWVWK
                  *: *:*  :*:***.::******** :***: :**::**:*: :  * :*::.: : :

seqA            MPFPM---
seqB            INLDKPRN
seqC            IDLGSN-
                : :
```

Fig. 2 (*continued*).

deletion mutant *lro1Δdga1Δ* and control when oleic acid serves as a carbon source (data not shown).

3.3. The contribution of TAG synthases to TAG accumulation depends on the carbon source

Cells of the single deletion mutants *lro1Δ* and *dga1Δ*, and the double deletion mutant *lro1Δdga1Δ* were grown to the stationary phase either on glucose or oleic acid containing medium, lipids of total cells extracted and analyzed for the neutral lipid composition (see Materials and methods). The single deletion mutant *lro1Δ* accumulates TAG to approx. 75% of wild-type level independent whether glucose or oleic acid serves as a carbon source (Fig. 4). In contrast, the amount of TAG is only significantly reduced in lipid extracts of *dga1Δ* cells grown on glucose containing medium (90% of control) but not when oleic acid serves as a carbon source. By growing cells of the double deletion mutant *lro1Δdga1Δ* on glucose containing medium the TAG level decreases to 30% of control. In contrast, in cell extracts of the double deletion mutant grown on oleic acid medium the amount of TAG is reduced to 85% of wild-type level. Since TAG are still formed in the double deletion mutant *lro1Δdga1Δ*, *Y. lipolytica* harbors at least one additional enzyme catalyzing the acylation of diacylglycerol.

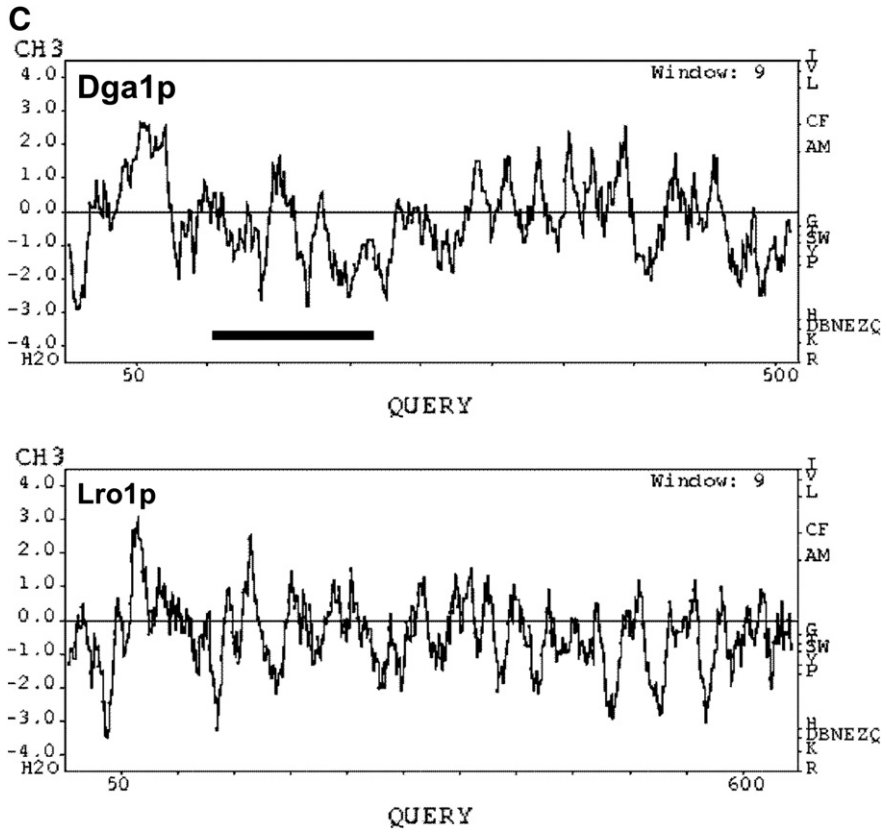

Fig. 2 (*continued*).

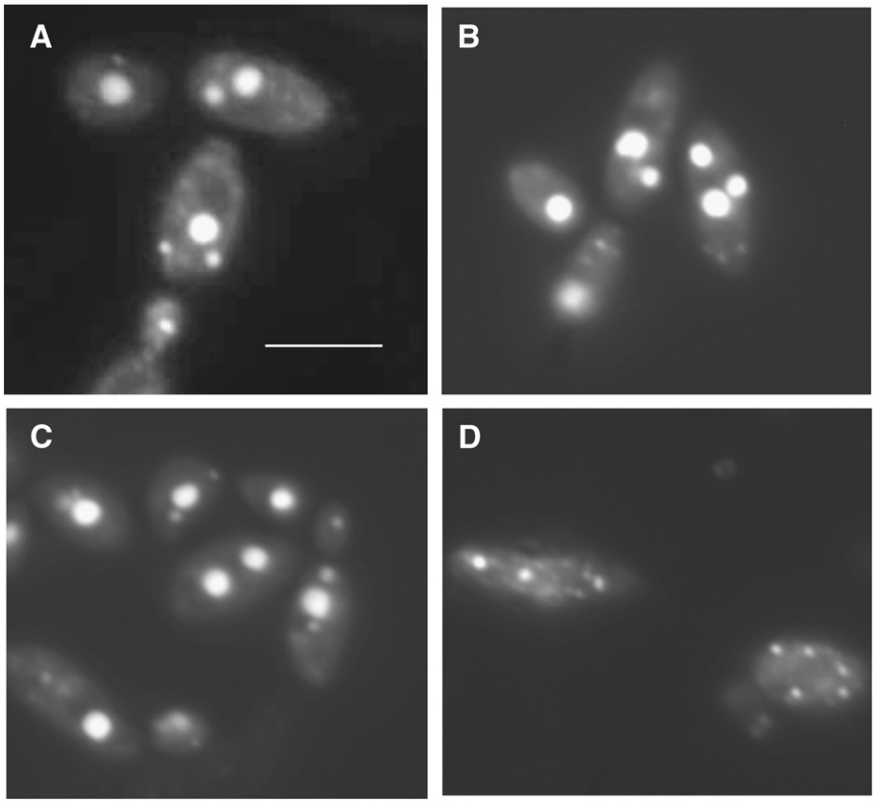

Fig. 3. Lipid particle formation in *Yarrowia lipolytica* mutants defective in triacylglycerol synthesis. Fluorescence microscopic inspection of wild-type cells (A), the single deletion mutants *lro1Δ* (B), *dga1Δ* (C) and the double deletion mutant *dga1Δlro1Δ* (D) grown to the stationary phase (24 h) on glucose containing media. Cells were stained with the fluorescent lipophilic dye NileRed®. Size bar: 5 μm.

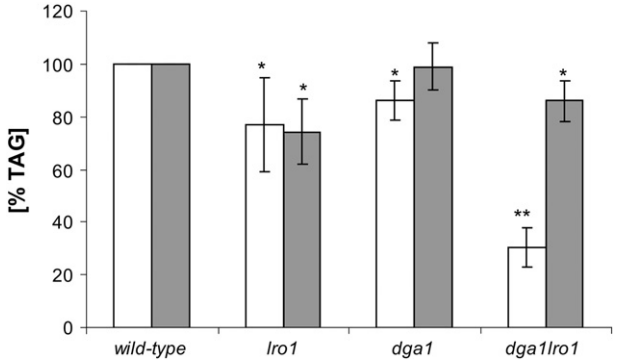

Fig. 4. Triacylglycerol accumulation in strains defective in triacylglycerol synthesizing enzymes. Accumulation of triacylglycerols (TAG) in the single deletion mutants *lro1Δ* , *dga1Δ* and the double deletion mutant *lro1Δdga1Δ* grown on glucose containing media (white bars) and on oleic acid containing media (gray bars). The value for wild-type (glucose: 0.71 ± 0.07% TAG/CDW; oleic acid: 17.9 ± 2.1% TAG/CDW) was set to 100%. Values are mean values of three independent experiments. *: P<0.05; **: P<< 0.0005; (Student's-*t*-test).

3.4. Lipid particle formation is restored upon heterologous expression of LRO1 and DGA1, respectively, in a Saccharomyces cerevisiae mutant otherwise lacking these cell compartments

Even though all results presented so far are pinpointing that Dga1p and Lro1p of the oleaginous yeast are indeed TAG synthases, it cannot be excluded that one of these proteins or both are involved in the regulation of TAG synthesis. Thus, heterologous expression experiments were performed.

As already mentioned above, neutral lipids such as TAG and steryl esters do not fit into membrane structures and are sequestered in lipid particles. In the budding yeast *S. cerevisiae* four enzymes catalyze the acylation of diacylglycerol yielding TAG, i.e., Lro1p, Dga1p, Are1p and Are2p [6]. The preferential substrates of Are1p and Are2p, however, are sterols. Since Are1p and Are2p are the only steryl ester synthases in the budding yeast, the quadruple mutant *lro1Δdga1Δare1Δare2Δ* lacks not only TAG but also steryl esters and consequently does not form lipid particles. To investigate the function of Dga1p and Lro1p of *Y. lipolytica* the respective genes were cloned into the vector pYES2 and heterologously expressed in the quadruple mutant *lro1Δdga1Δare1Δare2Δ* of *S. cerevisiae* (see Materials and methods). The *GAL1* promoter of the plasmid allows induction of gene expression by growing cells on galactose containing medium. Staining cells of both plasmid bearing *lro1Δdga1Δare1Δare2Δ* mutants with the lipophilic fluorescent dye NileRed® and microscopic inspection demonstrate the formation of lipid particles upon galactose induction (Fig. 5A, panels c and d). Whereas heterologous expression of *LRO1* in the quadruple deletion mutant background leads to the formation of lipid particles similar in size to the ones present in the corresponding *S. cerevisiae* wild-type strain, the size of the particles is significantly increased in the quadruple mutant expressing *DGA1*. The number of lipid particles formed upon heterologous expression of either *DGA1* or *LRO1* in the *lro1Δdga1Δare1Δare2Δ* quadruple mutant is limited to 1 to 2. As a negative control, no lipid particles are formed in the quadruple mutant *lro1Δdga1Δare1Δare2Δ* bearing the empty vector (Fig. 5A, panel b) or when transformants are grown on glucose containing minimal media (data not shown). Lipid analyses of total cells of the quadruple mutant *lro1Δdga1Δare1Δare2Δ* expressing either *DGA1* or *LRO1* of *Y. lipolytica* demonstrate the presence of TAG (Fig. 5C). The prominent TAG band in the lipid extract of the transformant expressing *DGA1* (Fig. 5C, lane 5) parallels the bigger size of lipid particles observed by fluorescence microscopy (Fig. 5A, panel c). The absence of steryl esters in lipid extracts of strains heterologously expressing *DGA1* or *LRO1* demonstrates that sterols do not serve as substrate of Dga1p and Lro1p (Fig. 5B).

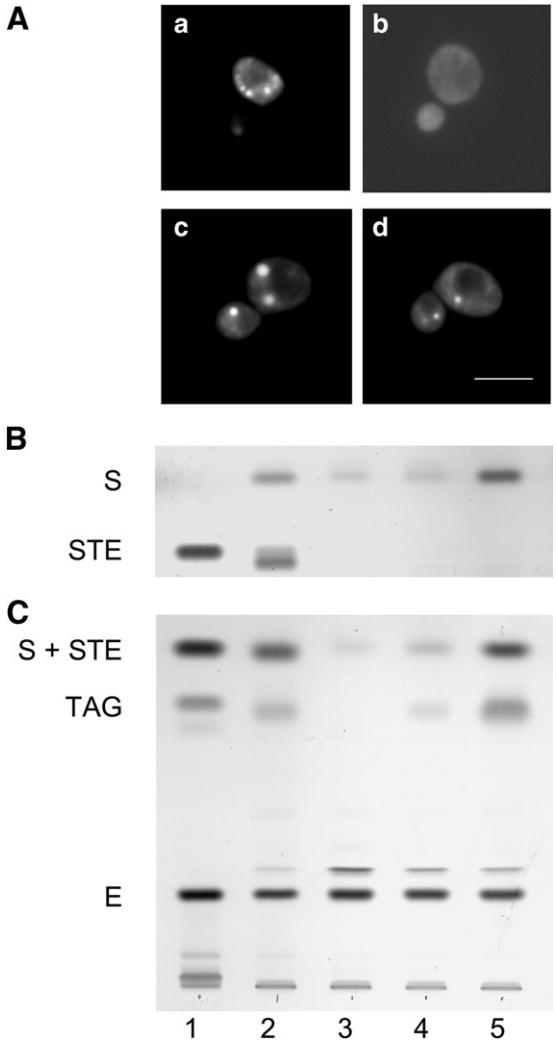

Fig. 5. Heterologous expression of *DGA1* and *LRO1*, respectively, restores the formation of triacylglycerols. (A) Fluorescence microscopic inspection reveals that lipid particles are formed upon heterologous expression of *DGA1* (c) and *LRO1* (d) in the quadruple mutant *lro1Δdga1Δare1Δare2Δ* of the budding yeast *Saccharomyces cerevisiae* otherwise lacking lipid particles (b). Panel a shows lipid particle formation in the corresponding wild-type strain of the quadruple mutant bearing the empty vector. Size bar: 5 μm. (C) Analysis of the lipid pattern of the quadruple mutant heterologously expressing either *LRO1* (lane 4) or *DGA1* (lane 5) and the quadruple mutant bearing the empty vector (lane 3). Lane 1: standard containing ergosterol (E), ergosteryl oleate (STE) and triolein (TAG); Lane 2: lipid extract of total cells of the corresponding wild-type strain bearing the empty vector. Solvent system: petroleum ether-diethyl ether-acetic acid 70:30:2 (per vol.). (B) To verify the absence of steryl esters in strains with quadruple mutant background lipids were additionally separated with a solvent system allowing the separation of squalene (S) and STE. Solvent systems: petroleum ether-diethyl ether-acetic acid (25:25:1; per vol.; development of the TLC plate to 1/3 of the distance) and petroleum ether-diethyl ether (49:1; vol./vol.; development to the full distance). Post-chromatographic staining was performed as described in Materials and methods. Note: The amounts of lipids separated by thin-layer chromatography were extracted from equal amounts of homogenates based on the protein content.

3.5. Lro1p and Dga1p use different acyl-donors for TAG synthesis

To elucidate the mechanism employed by Lro1p and Dga1p, respectively, for TAG formation enzyme assays specific for acyl-CoA dependent and acyl-CoA independent acylation of diacylglycerol were performed with transformants heterologously expressing *DGA1* or *LRO1* (see Materials and methods). In presence of oleoyl-CoA TAG synthase activity of homogenate of the *S. cerevisiae* quadruple mutant *lro1Δdga1Δare1Δare2Δ* heterologously expressing *DGA1* is 45-fold increased compared to the respective corresponding wild-type of the

quadruple mutant bearing the empty plasmid (Fig. 6A). However, in the assay specific for acyl-CoA dependent acylation some label is also incorporated into TAG when homogenate of *lro1Δdga1Δare1Δare2Δ* heterologously expressing *LRO1* serves as the enzyme source (60% of *S. cerevisiae* wild-type level). Since oleic acid is the most abundant fatty acid occurring in TAG of *Y. lipolytica* (52.5%) [12], enzymatic measurements for acyl-CoA dependent TAG synthesis were performed with oleoyl-CoA as a substrate. The second major fatty acid bound to the glycerol backbone of TAG is palmitic acid (22.1%). Comparison of the specific activities of Dga1p measured either in the presence of oleoyl-CoA or palmitoyl-CoA revealed a clear preference for the former fatty acid (479 ± 46 nmol/mg min vs. 108 ± 13 nmol/mg min).

By using phospholipids as acyl-donor microsomes of the quadruple mutant *lro1Δdga1Δare1Δare2Δ* containing *LRO1* of the oleaginous yeast incorporate 1.4 times more radioactive label into TAG than microsomes of the corresponding *S. cerevisiae* wild-type strain bearing the empty vector (Fig. 6B). Whereas substantial amounts of labeled TAG were formed in the presence of the phospholipid mixture used for the acyl-CoA independent assay (see Materials and methods), only traces of labeled TAG were formed when individual phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phophatidylserine) were used as acyl-donor. Especially phosphatidylcholine turned out to be a very poor substrate (data not shown). In the phospholipid:diacylglycerol acyltransferase specific assay microsomes were used as the enzyme source, because only traces of label were incorporated into TAG when homogenates of the respective strains were used as an enzyme source.

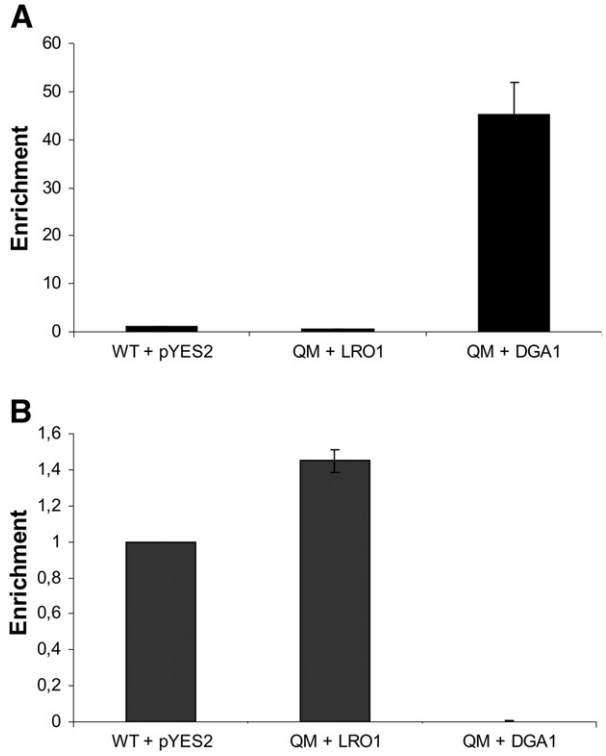

Fig. 6. Mechanism of triacylglycerol synthesis. Homogenates (A) or microsomes (B) of *Saccharomyces cerevisiae* wild-type cells bearing the empty vector (WT + pYES2) and the quadruple mutants *lro1Δdga1Δare1Δare2Δ* heterologously expressing *DGA1* (QM + *DGA1*) and *LRO1* (QM + *LRO1*), respectively, were used as enzyme sources in TAG synthase assays containing either oleoyl-CoA (A) or phospholipids (B) as a substrate. Activity of wild-type fractions was set to 1 (A_{spec} "WT + pYES2" with oleoyl-CoA as substrate (A): 10.2 ± 0.3 pmol/mg min; A_{spec} "WT + pYES2" with phospholipids as substrate (B): 11.8 ± 2.4 pg/mg min). Enrichment data were obtained from measurements of three independent data-sets. Note: In contrast to acyl-CoA independent TAG synthase activity (B), acyl-CoA dependent TAG synthase activity (A) is not restricted to microsomes (see Discussion). Thus, homogenates instead of microsomes were chosen as enzyme source in (A). No enzymatic activity was detected with the respective fractions of the quadruple mutant bearing the empty plasmid in the acyl-CoA dependent and acyl-CoA independent assay, which is in line with the lack of neutral lipids and lipid particles (see Fig. 5).

3.6. In vitro activities of acyl-CoA dependent and acyl-CoA independent TAG synthases in Yarrowia lipolytica depend on the carbon source used for cell growth

Results of heterologous expression experiments revealed that Dga1p catalyzes TAG synthesis via the acyl-CoA dependent pathway, whereas Lro1p prefers phospholipids as acyl-donor. To study TAG synthesis in *Y. lipolytica* in more detail homogenates of the single deletion strains *lro1Δ*, *dga1Δ*, the double deletion mutant *lro1Δdga1Δ* and the corresponding wild-type strain were used as an enzyme source in assays specific for either acyl-CoA dependent or acyl-CoA independent acylation of diacylglycerol (see Materials and methods). By using homogenate of the single deletion mutant *lro1Δ* (i.e., Dga1p is present) grown on glucose containing medium as the enzyme source TAG are only formed in the presence of oleoyl-CoA (Fig. 7A), but not when phospholipids serve as acyl-donor. In contrast, homogenate of *dga1Δ* cells (i.e., Lro1p is present) grown in the presence of glucose uses phospholipids as the preferred substrate for TAG synthesis. However, this enzyme source incorporates also a small but significant amount of radioactive label into TAG in the acyl-CoA dependent assay. The finding that under the same conditions homogenate of *lro1Δdga1Δ* cells grown on glucose containing medium lacks TAG synthase activity indicates that Lro1p of *dga1Δ* catalyzes the incorporation of the labeled fatty acid into TAG. This is in line with the observation that upon heterologous expression of *LRO1* in the *S. cerevisiae* mutant *lro1Δdga1Δare1Δare2Δ* homogenate of these cells forms TAG in the presence of acyl-CoA. Even though TAG are present in *lro1Δdga1Δ* cells of the oleaginous yeast grown on glucose containing medium (Fig. 4), in vitro homogenate of the respective mutant cells lacks also TAG synthase activity when phospholipids are provided as acyl-donor (Fig. 7A). In the budding yeast *S. cerevisiae* it has been shown that the contribution of Lro1p and Dga1p to TAG synthesis depends on the growth stage of the cells [5]. Whereas Lro1p is the major TAG synthase in cells of the exponential phase, TAG synthase activity of Dga1p is higher in cells entering the stationary phase. To test whether the same is true for Lro1p and Dga1p of *Y. lipolytica*, homogenate of wild-type cells of the exponential growth phase (OD_{600} ~ 1.2) and the stationary phase (OD_{600} ~ 5.4) were used as an enzyme source in the respective TAG synthase assays. Indeed, acyl-CoA independent TAG synthase (Lro1p) activity decreased by ~25% ($A_{spec} = 1.4 \pm 0.06$ pg/mg min vs. $A_{spec} = 1.1 \pm 0.05$ pg/mg min) and that of Dga1p increased by ~20% ($A_{spec} = 8.3 \pm 0.6$ pmol/mg min vs. $A_{spec} = 10 \pm 1.8$ pmol/mg min).

In analogy to the enzyme assays performed with homogenates of cells grown on glucose containing media, homogenates of cells grown on oleic acid containing media were tested for TAG synthase activities via both mechanisms (Fig. 7B). In the Lro1p-specific assay enzymatic activity of homogenate of *dga1Δ* cells (i.e., Lro1p is present) grown in the presence of oleic acid is 1.7-fold increased compared to wild-type. In contrast to homogenate of *lro1Δ* cells grown on glucose containing medium which lacks phospholipid: diacylglycerol acyltransferase activity (Fig. 7A), a small amount of label is incorporated into TAG via this pathway when the respective enzyme source of cells grown in the presence of oleic acid is used. However, under the same conditions homogenate of the double deletion mutant *lro1Δdga1Δ* is inactive. In the acyl-CoA dependent TAG synthase assay homogenates of both single deletion mutants and the double deletion mutant *lro1Δdga1Δ* form TAG, however, with lower efficiency than control. Noteworthy, in contrast to homogenate of *lro1Δdga1Δ* cells grown on glucose containing medium which do not incorporate the provided acyl-CoA group into TAG (Fig. 7A), homogenate of the respective mutant cells grown

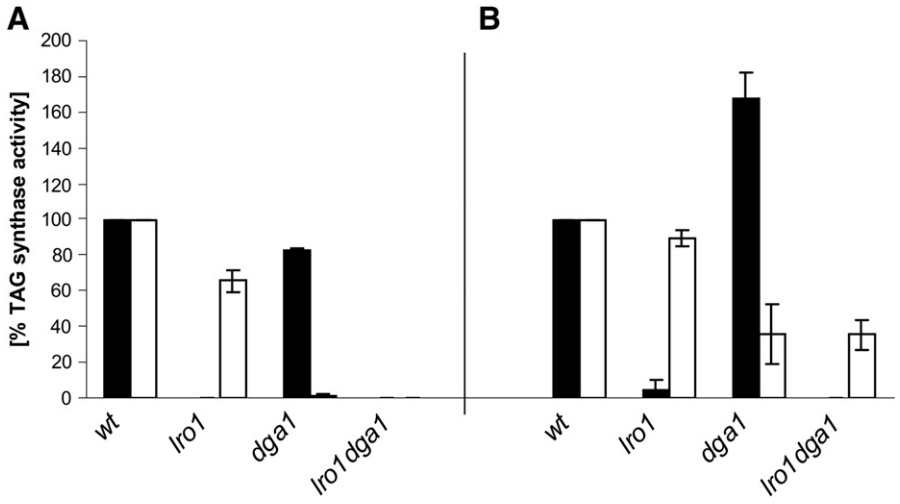

Fig. 7. In vitro triacylglycerol synthase activities of homogenates of cells defective in *DGA1* and/or *LRO1*. Homogenates of *Yarrowia lipolytica* cells grown either on glucose (A) or oleic acid (B) containing media were used as an enzyme source in triacylglycerol synthase assays specific for acyl-CoA independent (black bars) and acyl-CoA dependent (white bars) acylation of diacylglycerol (see Materials and Methods). The specific activity of wild-type samples was set to 100% (A_{spec} "wt" with oleoyl-CoA as substrate (white bars): 10.3 ± 1.8 pmol/mg min (glucose; A); 29.7 ± 1.5 pmol/mg min (oleic acid; B); A_{spec} "wt" with phospholipids as substrate (black bars): 1.1 ± 0.05 pg/mg min (glucose; A); 0.74 ± 0.1 pg/mg min (oleic acid; B); mean values of at least 3 independent experiments).

on oleic acid containing medium exhibit TAG synthase activity via the acyl-CoA dependent pathway. Since homogenates of both *dga1Δ* cells as well as *lro1Δdga1Δ* cells contain 35% of acyl-CoA dependent TAG-synthase activity compared to control (Fig. 7B), the observed acylation activity cannot be ascribed to Lro1p. Thus, in *lro1Δdga1Δ* cells grown on oleic acid containing medium at least one additional acyl-CoA dependent enzyme contributes to TAG synthesis.

4. Discussion

Here evidence is provided that the open reading frames YALI0E32769g and YALI0E16797g named hereafter *DGA1* and *LRO1*, respectively, encode major TAG synthases of the oleaginous yeast *Y. lipolytica*. Moreover, it is shown that growth on oleic acid containing medium induces acyl-CoA dependent TAG synthase activity catalyzed by an additional enzyme. This is concluded from data obtained by a combined biochemical, cell biological and molecular biological approach including lipid profiling of mutants deleted of *DGA1* and/or *LRO1*, measurements of enzymatic activity in vitro and heterologous expression experiments.

Recently, Ghosal et al. [21] demonstrated for Lro1p of the budding yeast *S. cerevisiae* that a transmembrane domain at the N-terminus is responsible for its localization to the endoplasmic reticulum (ER). Even though homology of phospholipid:diacylglycerol synthases of different yeast species is rather low at the N-terminal end, computational analysis predicts also for Lro1p of *Y. lipolytica* the presence of a transmembrane domain in this region (see Fig. 2C). The observation that heterologous expression of *LRO1* in a mutant of the budding yeast lacking TAG synthase activity not only restores TAG synthesis via the phospholipid: diacylglycerol acyltransferase mechanism but furthermore that the respective enzymatic activity is enriched in microsomes (ER) indicates that structural features rather than the N-terminal sequence are responsible for localization to the ER. Localization studies of Dga1p of the budding yeast revealed its presence in lipid particles and the ER [7]. A similar dual localization of Dga1p of *Y. lipolytica* is anticipated, because this polypeptide has formerly been identified as an unassigned lipid particle protein [12] and furthermore, acyl-CoA dependent TAG synthase activity is enriched in microsomes of a *Y. lipolytica* wild-type strain grown on glucose but absent in the respective enzyme source of *lro1Δdga1Δ* cells (K. Athenstaedt, unpublished result).

In *Y. lipolytica* TAG are a major component of so-called lipid particles, spherical structures sequestering neutral lipids from the cytosolic environment in a hydrophobic core [12]. Thus, the possibility of differences in lipid particle formation in cells defective in TAG synthesis has been considered. Microscopic inspection reveals that indeed in cells of the double deletion mutant grown on glucose containing medium the number and size of lipid particles are affected (see Fig. 3). These differences in lipid particle formation in *lro1Δdga1Δ* cells grown in the presence of glucose are paralleled by the lowest accumulation of TAG compared to the other strains tested (see Fig. 4). Since TAG serve as an energy source and/or a source of building blocks for membrane formation when nutrients are no longer provided by the environment, a smaller amount of TAG might lead to reduced long term viability and/or a prolonged lag phase after transferring the cells to fresh medium. However, neither any difference during the onset of growth nor in the long term viability (K. Athenstaedt, unpublished result) between *lro1Δdga1Δ* and wild-type has been observed, indicating that even the reduced amount of TAG in the double deletion mutant is sufficient for balanced growth.

In the budding yeast *S. cerevisiae* four enzymes contribute to TAG synthesis, namely Lro1p, Dga1p, Are1p and Are2p [6]. The quadruple mutant *lro1Δdga1Δare1Δare2Δ* is viable, lacks TAG as well as lipid particles, and has thus been chosen as a tool for studying the function of the polypeptides encoded by *DGA1* and *LRO1* of *Y. lipolytica*. Results of the heterologous expression experiments unambiguously identified Lro1p and Dga1p of the oleaginous yeast as TAG synthases. Moreover, these experiments revealed that whereas Dga1p requires acyl-CoA as a substrate for TAG synthesis, Lro1p uses phospholipids as acyl-donor. At the first sight the latter enzyme appears to accept at least to some extent acyl-CoA as a substrate as well, because in the TAG synthase assay specific for acyl-CoA dependent acylation significant amounts of TAG have been formed with homogenate of *lro1Δdga1Δare1Δare2Δ* cells heterologously expressing *LRO1* of the oleaginous yeast (see Fig. 6). However, in an additional experiment where the assay mixture contained radioactively labeled diacylglycerol as a substrate but no phospholipid substantial amounts of TAG were synthesized. This shows that phospholipids of the bilayer membrane containing Lro1p were efficiently used for TAG formation by this enzyme. Furthermore, acyl-CoAs are rapidly incorporated into phospholipids by a remodeling process. Thus, it is most likely that in

the respective assays the labeled acyl-CoA group ended up in TAG by an alternative route involving incorporation of the labeled fatty acid into phospholipids and subsequent transfer of the respective acyl-chain from those phospholipids surrounding Lro1p to TAG.

By using homogenate of the double deletion mutant *lro1Δdga1Δ* grown on glucose containing medium as an enzyme source no TAG is formed independent whether acyl-CoA or phospholipids is/are added as the respective acyl-donor (see Fig. 7A), thus indicating that Lro1p and Dga1p are the only TAG synthases of the oleaginous yeast. Lipid analysis (see Fig. 4) and fluorescence microscopic inspection (see Fig. 3), however, clearly demonstrate that the double deletion mutant *lro1Δdga1Δ* still forms TAG which are sequestered in lipid particles. Thus, *Y. lipolytica* harbors at least one additional enzyme besides Lro1p and Dga1p which catalyzes TAG formation. The fact that both types of in vitro assays do not yield TAG when homogenate of *lro1Δdga1Δ* cells grown on glucose containing medium serves as an enzyme source suggests that the potential additional TAG synthase catalyzes TAG formation by a different mechanism, probably by converting two diacylglycerol molecules to TAG and monoacylglycerol (see Fig. 1). However, since isoenzymes do not necessarily require the same conditions for revealing enzymatic activity and/or may be active at different growth phases, the additional TAG synthase(s) may nevertheless form TAG by a mechanism similar to Dga1p and Lro1p.

The scenario of TAG synthesis in *Y. lipolytica* cells significantly changes when oleic acid serves as the carbon source (see Figs. 4 and 7B). In *lro1Δ* cells TAG accumulate to approximately 75% of wild-type level independent whether glucose or oleic acid serves as the carbon source. In contrast, the ratio of TAG accumulation in mutants defective in *DGA1* to wild-type increases in favor of the mutant when oleic acid serves as a carbon source. Whereas the increased ratio of TAG accumulation in *dga1Δ* cells can be explained by a higher TAG synthase activity via the phospholipid:diacylglycerol acyltransferase specific pathway, another reason has to be relevant for the higher ratio of TAG accumulation in *lro1Δdga1Δ* cells. Results of in vitro experiments clearly demonstrate that in *lro1Δdga1Δ* cells growing on oleic acid medium at least one additional acyl-CoA dependent enzyme contributes to TAG synthesis. Since this additional TAG synthase reveals its enzymatic activity especially in cells lacking both Lro1p and Dga1p it is hypothesized that the respective enzyme may function as a kind of security system preventing the accumulation of toxic amounts of fatty acids. A toxic effect of higher amounts of free fatty acids is anticipated, since mutant cells of the fission yeast *S. pombe* lacking TAG synthesis undergo apoptosis upon reaching the stationary phase which is paralleled by accumulation of free fatty acids and diacylglycerols [11]. Similarly, cell viability is strongly decreased in the quadruple mutant *lro1Δdga1Δare1Δare2Δ* of the budding yeast *S. cerevisiae*, likewise defective in TAG formation, when oleic acid serves as a carbon source [22].

The future task for understanding the complex process of TAG synthesis in the oleaginous yeast *Y. lipolytica* will be the identification and characterization of additional polypeptides governing TAG synthesis. Especially the identification of the additional acyl-CoA dependent TAG synthase specifically induced upon growth on oleic acid containing medium will be a major step forward to understand the complexity of TAG metabolism in the oleaginous yeast. Since the ability of *Y. lipolytica* to accumulate huge amounts of TAG in the presence of fatty acids resembles adipocytes of higher eukaryotes, the knowledge about TAG metabolism in this yeast may provide useful information for similar studies in higher eukaryotes where defects in lipid turnover are related to several severe diseases such as obesity, arteriosclerosis and diabetes.

Acknowledgement

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